REMARKS

This document is filed in response to the Office Action dated November 26, 2010 ("Office Action").

Applicants have amended claims 20, 25, 28, 29, 34, and 37 to more particularly point out the subject matter that they deem as their invention. Support for the amendments to claims 20, 25, 28, 34, and 37 can be found in the Specification at page 23, lines 2 and 3, as well as in Figure 6. Support appears for the amendment to claim 29 at page 41, lines 3-11.

Further, Applicants have amended claims 18, 21, 23, 26, 30, 32, and 35 to promote clarity.

No new matter has been added by the amendments to the claims.

Claims 18-37 are currently pending and under examination. Note that claims 1-17 and 38-51 were previously cancelled.

Applicants respectfully request that the Examiner reconsider this application in view of the remarks below.

Objections to the Claims

The Examiner objected to claims 18, 23, and 26 for informalities. More specifically, he asserted that the phrase "in a target organ **in** the restriction enzyme-recognizing unit" recited in these claims should read "in a target organ **into** the restriction enzyme-recognizing unit." See the Office Action, page 4, third paragraph.

Applicants have amended claims 18, 23, and 26 to recite "in a target organ, **into** the first and second multiple cloning sites" and respectfully request withdrawal of this objection.

The Examiner also objected to confusing language in claim 26. See the Office Action, page 4, fourth paragraph.

This claim, as mentioned above, has been amended for clarity. Applicants respectfully request that the Examiner also withdraws this objection.

Rejection under 35 U.S.C. § 112, second paragraph

The Examiner rejected claims 18-37 for indefiniteness on four grounds, which will be discussed separately below.

I.

The Examiner deemed claims 18, 23, and 26 as indefinite, asserting that the phrase "the protein-coding gene," recited in these claims, lacked proper antecedent basis. See the Office Action, page 2, fifth paragraph. It is the Examiner's position that the phrase at issue improperly refers to a specific gene in the adenovirus E1B coding region, i.e., the E1B-19K or E1B-55K gene.

Applicants have replaced that phrase in claims 18, 23, and 26 with the phrase "a protein-coding gene." Clearly, with the replacement, an antecedent basis for this phrase is no longer necessary.

It follows that claims 18, 23, and 26, as amended, are now definite with respect to this ground for rejection.

Claims 19-22, depend from claim 18, claims 24, 25, and 32-37 depend from claim 23, and claims 27-31 depend from claim 26. These claims were deemed by the Examiner as indefinite for depending from rejected claims 18, 23, and 26. As set forth above, claims 18, 23, and 26, as amended, are definite. So are the claims that depend from them.

II.

The Examiner rejected claims 18, 23, and 26, alleging that certain steps in the claimed methods were unclear. More specifically, he stated that "... it is unclear into which of the three restriction sites or sequences ... the promoter is to be introduced into." See the Office action, page 3, lines 1 and 2.

Applicants have amended these claims to replace the phrase "restriction enzyme-recognizing unit" with the phrase "expression cassette," as suggested by the Examiner. Applicants have also amended these claims to clearly set forth a step in which a first and a second promoter are inserted into a first and a second multiple cloning site, respectively. Applicants submit that claims 18, 23, and 26, as amended, are definite.

Claims 19-22, 24, 25, and 27-37, rejected on the same ground as these claims, are also definite for at least the reasons set forth above.

III.

The Examiner also rejected claim 26 for not reciting a step for preparing an adenoviral vector as set forth in the claim preamble. See the Office Action, page 3, second paragraph.

Applicants point out that claim 26, as amended, includes a step in which a proliferation-regulated plasmid is integrated into a plasmid containing an adenoviral genome to yield a proliferation-regulated adenoviral vector. See claim 26, step (a)(ii).[1] In a subsequent step, a therapeutic gene is inserted into the proliferation-regulated adenoviral vector. Clearly, performing the steps recited in claim 26 would result in production of a proliferation-regulated adenoviral vector that includes a therapeutic gene, as recited in its preamble.

IV.

Additionally, the Examiner rejected claims 23 and 26 for using the term "high-expression" when referring to a constitutive promoter, asserting that this is a relative term not defined in the Specification. See the Office Action, page 4, second paragraph.

Applicants have amended these claims to recite "constitutive strong promoter." This phrase is well known in the art and clearly refers to a promoter that is always active and that leads to elevated expression levels of a gene linked to it. See, e.g., claim 8 in US Patent 5,891,693, a copy of which is attached hereto as Exhibit A.

According to MPEP 2173.02, to be considered definite, "... claims [must] set out and circumscribe particular subject matter with a **reasonable degree of clarity and particularity**."

Applicants posit that a skilled artisan would know whether a particular promoter is a strong promoter or not. In view of the well known use of the term strong promoter, claims 23 and 26, as amended, are definite and would be understood by a skilled person

[1] Note that step (a)(ii) of claim 26 has not been amended in the current response, as, contrary to the Examiner's assertion, this claim did include a step for making an adenoviral vector prior to the current amendment. As mentioned above, the claim has been amended solely to promote clarity.

in the art. In other words, these claims set out particular subject matter with a **reasonable degree of clarity and particularity**, as required by MPEP 2173.02.

V.

For the reasons set forth above, Applicants submit that claims 18-37 are definite and the rejection should be withdrawn.

Double Patenting Rejection

The Examiner contended that claims 20, 25, 28, 34, and 37 "merely recite all possible [limitations] already found in the parent claims[, i.e., claims 18, 23, or 26]." See the Office Action, page 5, lines 4-6. More specifically, he asserted that both the parent claims and their dependents cover both possible variations of E1B expression, i.e., E1B-19K or E1B-55K. *Id.*

Applicants have amended claims 20, 25, 28, 34, and 37 such that they cover only the E1B-19K protein. As these claims now no longer "recite all possible limitations already found in the parent claims," the rejection has been overcome and withdrawal thereof is requested.

CONCLUSION

It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue or comment does not signify agreement with or concession of that rejection, issue or comment.

In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed.

Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, except as specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

The Petition for Extension of Time fee in the amount of $245.00 is being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account

authorization. Please apply any other charges or credits to Deposit Account No. 50-4189, referencing Attorney Docket No. 55801-002US1.

Respectfully submitted,

Date: 4-26-11

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Exhibit A

US005891693A

United States Patent [19]

Bebbington et al.

[11] Patent Number: 5,891,693

[45] Date of Patent: Apr. 6, 1999

[54] RECOMBINANT DNA METHODS VECTORS AND HOST CELLS

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[73] Assignee: Alusuisse Holdings A.G., Neuhausen am Rheinfall, Switzerland

[21] Appl. No.: 376,380

[22] Filed: Jan. 23, 1995

Related U.S. Application Data

[63] Continuation of Ser. No. 898,165, Jun. 12, 1992, which is a continuation of Ser. No. 460,154, Jan. 25, 1990, abandoned.

[51] Int. Cl.[6] C12N 15/00; C12P 21/06; C12P 21/04; C07H 21/02

[52] U.S. Cl. 435/172.3; 435/320.1; 435/69.1; 435/696; 536/23.1

[58] Field of Search 435/172.3, 320; 536/23.1

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Attorney, Agent, or Firm—Spencer & Frank

[57] ABSTRACT

The present invention relates to vectors useful for transforming a lymphoid cell line to glutamine independence. The vectors comprise an active glutamine synthetase (GS) gene as well as a heterologous gene of interest to be expressed. The preferred embodiments encompass vectors wherein the heterologous gene is expressed from a relatively strong promoter and the GS gene is expressed from a relatively weak promoter. In one example, the heterologous gene is operatively linked to the hCMV-MIE promoter and the GS gene is operatively linked to the SV40 early region promoter.

25 Claims, 3 Drawing Sheets

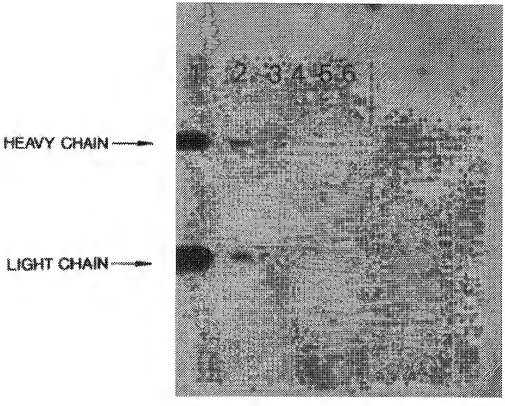


Fig. 1

Fig. 2

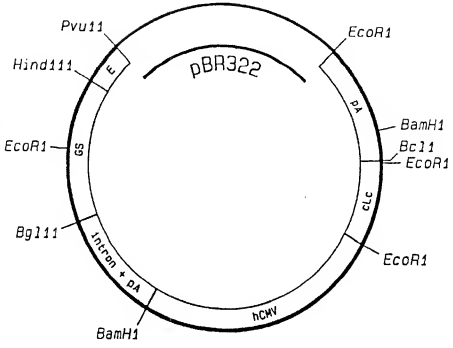

Pvu11
Hind111
EcoR1
BamH1
Bcl1
EcoR1
EcoR1
Bgl11
BamH1
pBR322
E
GS
PA
CLC
intron + PA
hCMV

Fig. 3

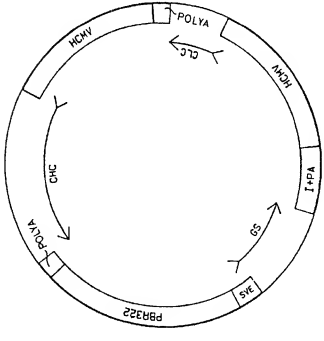
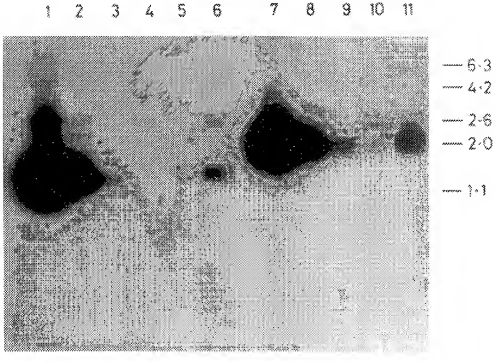

POLYA
CLC
HCMV
HCMV
I-PPA
GS
3'G
PBR322
POLYA
CMC

Fig. 4



RECOMBINANT DNA METHODS VECTORS AND HOST CELLS

This is a continuation of application Ser. No. 07/898,165, filed Jun. 12, 1992, which is a continuation of application Ser. No. 07/460,154 filed Jan. 25, 1990, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods for improving the usefulness of lymphoid cell lines as host cells for the production of proteins by recombinant DNA technology. The present invention also relates to vectors for use in such methods and to host cells produced by such methods.

2. Description of the Prior Art

Lymphoid cell lines are at present being appraised for use as host cells in the production by recombinant DNA technology of immunoglobulin molecules, related hybrid or chimeric proteins (Ig-type molecules), or other recombinant proteins. Since the lymphoid cells include myeloma cells which are of the same general type as the B cells which produce Ig molecules in vivo, it is envisaged that they will naturally possess the intracellular mechanisms necessary to allow proper assembly and secretion of Ig-type molecules. Such lymphoid cell lines may also be of use in the production by recombinant DNA technology of non-Ig-type molecules.

It is known that many lymphoid cell lines, such as myeloma cell lines and T cell lymphomas, cannot be grown in vitro on media lacking in glutamine. It has been suggested that it would be useful to be able to transform lymphoid cell lines to glutamine independence, since this may provide an advantageous method for selecting transformed cell lines.

It has been conjectured that such a cell line could be transformed to glutamine independence by incorporating therein a gene coding for glutamine synthetase (GS). Such a suggestion is made in EP-A-0 256 055 (Celltech). However, it has subsequently been found that hybridoma cell lines can generate spontaneous variants able to grow in a glutamine-free medium at such a high frequency that the identification of transfectants is difficult or impossible. For myeloma cell lines, transfection with a GS gene and growth of the transformed cells in a glutamine-free medium does not result in significant survival rates.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for transforming lymphoid cell lines to glutamine independence.

According to the present invention, there is provided a method for transforming a lymphoid cell line to glutamine independence which comprises:

transforming the lymphoid cell line with a vector containing an active glutamine synthetase (GS) gene;

growing the transformed cell line on a medium containing glutamine; and

continuing the growth of the transformed cell line on a medium in which the glutamine is progressively depleted or on a medium lacking glutamine.

Preferably, the lymphoid cell line is a myeloma cell line.

Preferably, the glutamine-depleted or glutamine-free medium contains asparagine. Alternatively the medium contains another nutrient which enables the transformed cell line to survive on a glutamine free medium. This other nutrient may be an ammonia donor, such as ammonium chloride.

It has surprisingly been found that if the transformed lymphoid cell line is not firstly grown on a glutamine-containing medium, it is not possible to obtain the growth of any cell line, whether or not it has been transformed by the vector. By use of the method of the present invention, it is possible to select for lymphoid cell lines which have been transformed by the vector.

Alternatively, the lymphoid cell line may be transformed with a vector containing both an active GS gene and a gene encoding another selectable marker, such as a gpt gene, or cotransformed with separate vectors encoding GS and the selectable marker respectively. Transformed host cells can then be selected using the selectable marker prior to depletion of glutamine in the medium.

The advantage of this method is that it enables selection for vector maintenance to be achieved without the use of a toxic drug. Host cells in which the vector is eliminated will not be able to survive in a glutamine-free medium.

A further advantage of this method is that it enables selection for gene amplification to be carried out without the risk of amplification of the host cell's endogeneous GS genes.

Preferably the glutamine in the medium is progressively depleted by dilution with a medium containing aspargine but lacking glutamine.

Preferably, the vector used to transform the lymphoid cell line also contains an active gene coding for a protein heterologous to the lymphoid cell line. Alternatively, the lymphoid cell line may be co-transformed with a separate vector containing the active gene coding for the heterologous protein.

The heterologous protein may be one which is expressed as a single chain (although it may be cleaved after expression into a multichain protein). Examples of such single chain expression products are tissue plasminogen activator (tPA), human growth hormone (hGH) or tissue inhibitor of metalloproteinase (TIMP).

Preferably, however, the heterologous protein is an Ig-type molecule. Such molecules require the separate expression of two peptide chains which are subsequently assembled to form the complete molecule. Thus, the cell line will need to be transformed with active genes which encode separately a heavy chain (or heavy chain analog) and a light chain (or light chain analog).

Preferably, the genes encoding the heavy and light chains are both present on the same vector as the GS gene. Alternatively, the vector containing the GS gene may have one of the heavy or light chain genes thereon, the other gene being on a separate vector. In a second alternative, the light and heavy chain genes are not present on the vector containing the GS gene but are present on the same or different vectors.

The expression of such heterologous proteins may be substantially increased by subsequent selection for GS gene amplification, for instance using methionine sulphoximine (MSX) as the selection agent.

It is preferred that the GS gene comprises a relatively weak promoter and that the gene (or genes) encoding the heterologous protein comprises a relatively strong promoter so that in the transformed cell lines, protein synthesis is directed preferentially to the production of the heterologous protein or peptide rather than to the production of GS. Moreover, a lower concentration of selection agent, such as MSX, will be required to select for gene amplification if the GS gene is controlled by a weak, rather than a strong, promoter.

It is also conjectured that use of a weak promoter may enable the selection of transformed cell lines wherein the GS

gene has been inserted at a particularly advantageous location in the genome. This will ensure that both the GS gene and any heterologous genes will be transcribed efficiently.

It has been found that, in the preferred case, where all the genes are present on the same vector, it is necessary to design the vector carefully in order to achieve proper expression of the genes.

Thus, according to a second aspect of the present invention, there is provided a vector for transforming a lymphoid cell line to glutamine independence and to enable it to produce a heterologous protein, the vector comprising a GS gene and a gene encoding the heterologous protein, wherein the vector is arranged such that expression of the GS gene is not hindered by transcriptional interference from the promoter/enhancer transcribing the sequence coding for the heterologous protein to such an extent that glutamine-independent colonies cannot be produced.

Preferably, the genes on the vector are arranged in such orientations and with such promoters as substantially to prevent transcriptional interference. For instance, the GS gene may contain a relatively weak promoter, the gene encoding the heterologous protein may contain a relatively strong promoter, and the promoter of the GS gene may be located upstream of or may direct expression in the opposite direction to that of the gene encoding the heterologous protein.

It has surprisingly been found that if the vector arrangement set out above is adopted, the GS gene is expressed in sufficient quantity to enable selection to be made and the heterologous protein is expressed more efficiently than with other vector arrangements.

It has been observed that other vector arrangements, for instance using different promoters or a different ordering or orientation of the genes, can lead to a much reduced or even non-existent level of GS or heterologous protein production. It is conjectured (although the applicants do not wish to be limited to this theory) that if a gene containing a strong promoter is located upstream of a GS gene having a weaker promoter, the transcription of the upstream gene will run through into the downstream gene, thus producing occlusion of the downstream promoter. Since the frequency of transformed colonies is critically dependent on the level of GS gene expression, such promoter occlusion dramatically reduces the frequency with which transfectants are recovered.

A preferred combination for the weak and strong promoters is the SV40 early region and the hCMV-MIE promoters. (hCMV-MIE=human cytomegalowirus major immediate early gene). However, other suitable promoter combinations will be readily apparent to those skilled in the art.

A particularly preferred embodiment of the vector of the present invention comprises a GS gene having a weak promoter having downstream therefrom a heavy chain-like gene having a strong promoter, there being on the vector a light chain-like gene having a strong promoter oriented in the opposite direction to the promoters of the GS and heavy chain-like genes.

Alternatively, promoter occlusion may be prevented by use of transcription terminator signals between the genes.

In another alternative, the genes may be arranged with a unique restriction site between them. This site can then be used to linearise the vector before it is incorporated into the host cell. This will ensure that in the transformed host cell no promoter occlusion can take place.

It will be appreciated that if the vector contains more than one gene encoding a heterologous protein, it will be necessary to ensure that none of the genes in the vector can promote transcriptional interference. For instance, if the vector contains a GS gene, a heavy chain gene and a light chain gene, it is preferred that either all three genes are transcribed in the same direction and that the GS gene is upstream of the other two genes or that the GS gene and one of the other genes are transcribed in the same direction, the GS gene is upstream of the first other gene, and the second other gene is transcribed in the other direction, and the promoter of the second other gene is located adjacent the promoter of the GS gene.

The vector may comprise a viral vector, such as lambda phage, or a plasmid vector, for instance based on the well known pBR322 plasmid. However, any other of the vectors well known in the art may be adapted by use of conventional recombinant DNA technology for use in the present invention.

The present invention also includes host cells produced by the method of the invention or containing vectors according to the invention.

In particular, the present invention includes a lymphoid cell line which has been cotransformed with a vector containing a GS gene and a vector containing a gene encoding a heterologous protein, the vectors being arranged to ensure that the GS gene is not hindered by transcriptional interference to such an extent that glutamine-independent colonies cannot be produced.

The present invention is described below by way of example only with reference to the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows an analysis of proteins secreted by NSO cells transfected with plasmid pAB2GS by Western blotting in which 25 μl of culture supernatant or control tissue culture medium was run on a 10% SDS reducing polyacrylamide gel, blotted onto nitro-cellulose and probed with antisera recognising human Ig chains and then with ^{125}I-labelled protein A;

FIG. **2** shows the structure of plasmid pSV2GScLc;

FIG. **3** shows the structure of plasmid pST6; and

FIG. **4** shows a Southern blot analysis of genomic DNA from cell lines SV2GSNSO and CMGSNSO.

In FIG. 1 of the drawings lane 1 shows purified chimeric B72.3 antibody to show the position of lg light and heavy chains, lanes 2 to 5 show culture supernatants from four different transfected clones, and lane 6 shows culture medium as a negative control.

In FIG. **2**, E is the SV40 early region promoter, GS is a GS cDNA coding sequence, intron+PA is the small t-intron and the early region polyadenylation signal of SV40, BCMV is the hCMV-MIE promoter-enhancer, CLC is the coding sequence for the chimeric L-chain of a humanised antibody known as B72.3, and pA is the SV40 early polyadenylation signal.

In FIG. **3**, hCMV is the hCMV-MIE promoter enhancer (2.1 kb) fragment. CHC is the chimeric heavy chain coding sequence of the B72.3 antibody. CLC is the chimeric light chain coding sequence of the B72.3 antibody. Poly A contains the SV40 early polyadenylation signal. 1+PA contains the small t intron of SV40 and the early region polyadenylation signal. SVE is the SV40 early promoter. A bacterial plasmid origin of replication and ampicillin resistance gene are provided by pBR322.

FIG. **4** shows a copy number analysis of GS-vectors in NSO cells before and after selection with MSX. DNA samples were digested with BglI and BglII, electrophoresed

on a it agarose gel, transferred to nitrocellulose and probed with the 0.5 kb 5' Pst1 DNA fragment of pGSC45 [7] isolated from a GS cDNA.
DNA samples are as follows:

Lane 1	plasmid pSV2GS equivalent to 300 copies/cell
Lane 2	plasmid pSV2GS equivalent to 10 copies/cell
Lane 3	plasmid pSV2GS equivalent to 1 copy/cell
Lane 4	10 μg NSO genomic DNA
Lane 5	10 μg SV2GSNSO genomic DNA
Lane 6	10 μg SV2GSNSO (100 μM MSX resistant) genomic DNA
Lane 7	plasmid pCMGS equivalent to 300 copies/cell
Lane 8	plasmid pCMGS equivalent to 10 copies/cell
Lane 9	plasmid pCMGS equivalent to 1 copy/cell
Lane 10	10 μg CMGSNSO genomic DNA
Lane 11	10 μg CMGSNSO (100 μM MSX resistant) genomic DNA
m.w.	λ phage DNA digested with ClaI; molecular weight markers

A list of references is given at the end of the description. In the following, the references are indicated by numbers enclosed in square brackets.

VECTORS

In the following Examples, for comparative purposes, two plasmids described in EP-A-0 256 055 were used. These are plasmids pSVLGS1 and pSV2GS. Plasmid pSVLGS1 contains a GS minigene, containing cDNA and genomic DNA sequences, under the control of a SV40 late region promoter. Plasmid pSV2GS contains a cDNA sequence encoding GS under the control of a SV40 early region promoter.

A vector pSV2BamGS was produced by converting the unique PvuII site in pSV2GS to a BamHI site by the addition of a synthetic oligonucleotide linker.

By use of synthetic oligonucleotide linkers, the major immediate early gene promoter, enhancer and complete 5'-untranslated sequence from human cytomegalovirus (hCMV-MIE) (the Pst-1m fragment [1] together with a synthetic oligonucleotide to recreate the remaining 5' untranslated sequence) was inserted between the NcoI sites of pSV2GS such that the hCMV-MIE promoter directs expression of the GS coding sequence. The resulting plasmid was labelled pCMGS.

Plasmid pSV2BamGS was digested with BamHI to give a 2.1 kb fragment containing the transcription cassette.

For convenient construction of other expression plasmids, a basic vector pEE6 was used. Plasmid pEE6 contains the XmnI to BclI fragment of plasmid pCT54 [2] with the polylinker of plasmid pSP64 [3] inserted between its HindIII and EcoRI sites but with the BamHI and SALI sites removed from the polylinker. The BclI to BamHI fragment is a 237bp SV40 early gene polyadenylation signal (SV40 nucleotides 2770–2533). The BamIII to BglI fragment is derived from plasmid pBR322 [4] (nucleotides 275–2422) but with an additional deletion between the SalI and AvaI sites (nucleotides 651–1425) following addition of a SalI linker to the AvaI site. The sequence from the BglI site to the XmnI site is from the β-lactamase gene of plasmid pSP64 [3].

Plasmid pEE6gpt contains the transcription unit encoding xanthine-guanine phosphoribosyl transferase (gpt) from plasmid pSV2gpt [5] cloned into plasmid pEE6 as a BamHI fragment by the addition of a BamIII linker to the single PvuII site of plasmid pSV2gpt.

By similar means, a derivative of plasmid PCMGS containing the transcription cassette for the xanthine-guanine phosphoribosyl transferase (gpt) gene from pEE6gpt was produced. The plasmid thus produced was labelled pCMGS-gpt.

Plasmid pEE6hCMV contains the hCMV-MIE promoter-enhancer and complete 5' untranslated sequence inserted by means of oligonucleotide linkers into the HindIII site of plasmid pEE6.

Plasmid pEE6hCMVBglII is a derivative of pEE6hCMV in which the HindIII site upstream of the hCMV enhancer has been converted to a BglII site by blunt-ending and addition of a synthetic oligonucleotide linker.

Plasmid pEE6HCLCBg is a vector derived from pEE6hCMV containing a coding sequence for a mouse-human chimeric Ig light chain from the B72.3 antibody [6] inserted into the EcoRI site of pEE6hCMV such that the light chain is under the control of the hCMV-MIE promoter-enhancer. (The upstream HindIII site has also been converted to a BglII site by standard methods.) The 2.1 kb BamHI fragment from pSV2BamGS was inserted into pEE6HCLCBg to produce a plasmid pcLc2GS in which the Ig light chain and GS genes are transcribed in the same orientation with the GS gene downstream of the light chain gene.

pEE6HCHHCL is a vector which contains sequences coding for both the heavy and light chains of the chimeric B72.3 antibody [6] under the control of hCMV-MIE promoter enhancers. The 2.1 kb BamHI fragment from pSV2BamGS was inserted into pEE6HCHHCL to produce a plasmid pAb2GS in which the heavy and light chain genes and the GS gene are all transcribed in the same orientation in the order heavy chain, light chain, GS.

A 3.1 kb BglII-BamHI fragment from pEE6HcLcBg was inserted into the BamHI site of pSV2GS to produce a plasmid pSV2GScLc in which the chimeric light chain gene and the GS gene are transcribed in the same orientation with the GS gene upstream of the light chain gene.

Similarly, the 3.1 kb BglII-BamHI fragment of PEE6HCLCBg was inserted into the BamHI site of pCMGS to produce a plasmid pCMGS.CLC in which both genes are again in the same orientation.

pEE6CHCBg is a plasmid containing the heavy chain gene of chimeric B72.3 antibody [6] under the control of the hCMV-MIE promoter-enhancer and SV40 polyadenylation signal. The hCMV-MIE chain termination unit was excised from the plasmid as a 4.7 kb partial HindIII-BamHI fragment and inserted, by means of a HindIII-BamHI oligonucleotide adaptor, at the single BamHI site of psv2GScLc to form pSV2GScLccHc. The BamHI site upstream of the hCMV-MIE-cH chain transcription unit in pSV2GScLccHc was then removed by partial BamHI digestion, filling in with DNA polymerase 1 and religating to form pST6.

A gene coding for a novel fibrinolytic enzyme of 90 kD molecular weight was isolated as a 2.8 kb HindIII to BglII fragment. This was then inserted between the HindIII and BclI sites of the expression plasmid pEE6hCMVBglII in the appropriate orientation such that the hCMV promoter directed transcription of the inserted gene. An SV40 Early-GS transcription unit was excised as a BamHI fragment from pSV2GS and inserted into the BglII site at the 5' end of the hCMV sequence in pEE6hCMVBglII, in the appropriate orientation such that transcription from the hCMV promoter and the SV40 early promoter is in the same direction. This formed the plasmid pEE690KGS.

Cell Lines

In the Examples, the following cell lines were used: NSO and P3-X63Ag8.653, which are non-producing variants of the mouse P3 mouse plasmacytoma line; Sp2/0, which is a non-producing mouse hybridoma cell line; and YB2/0, which is a non-producing rat hybridoma cell line.

Media

All cells were grown in either non-selective medium, Dulbecco's Minimum Essential Medium (DMEM) containing 2 mM glutamine, 100 μM non-essential amino acids, 10% foetal calf serum and streptomycin/penicillin, or in glutamine-free DMX (G-DMEM) containing 500 μm each of glutamate and asparagine, 30 μM each of adenosine, quanosine, cytidine and uridine, 10 μM thymidine, 100 μM non-essential amino acids, 10% dialysed foetal calf serum and streptomycin/penicillin, or in derivatives of G-DMEM lacking various of these additives.

Alternatively, cells were cultured in gpt-selective media, made using the following filter-sterilised stock solutions: 1) 50× each of hypoxanthine and thymidine; 2) 50× xanthine (12.5 mg/ml in 0.2M NaOH); 3) mycophenolic acid (MPA, 250 μg/ml in 0.1M NaOH); and 4) 1M HCl. gpt-selective medium is made by mixing 93 ml of non-selective medium (described above), 2 ml solution 1), 2 ml solution 3) and 0.6 ml solution 4). 2× gpt is made by mixing 86 ml of non-selective medium with twice the above quantities of solutions 1) to 4).

Linearisation of Plasmids

In order to introduce them into cells all plasmids were linearised by digestion with an appropriate restriction enzyme which cuts at a single site in the plasmid and hence does not interfere with transcription of the relevant genes in mammalian cells. Typically 40 μg of circular plasmid was digested in a volume of 400 μl restriction buffer. The enzymes used for linearisation of the plasmids are shown in Table 1.

TABLE 1

Enzymes used for Linearisation of plasmid	
Plasmid	Restriction Enzyme
PSVLGS.1	PvuI
pSV2.GS	PvuI
pSV2.Sam GS	PvuI
pCMGS	PvuI
pCMGS.gpt	PvuI
PEE6.gpt	SalI
pcLcZGS	SalI
pAb2GS	SalI
pSV2.GSCLc	TthIII
pCMGS.cLc	TthIII
pST6	BamHI
pEE690KGS	SalI

Electroporation of Cells

Cells were harvested while growing exponentially, washed once in phosphate-buffered saline (PBS) by centrifugation at 1200 rpm in a bench centrifuge and resuspended at a density of 10^7 cells/ml in fresh ice cold PBS. One ml of cell suspension was added to the digested plasmid DNA (0.4 ml in restriction buffer) and incubated on ice for 5–10 minutes. The cell-DNA mixture was then subjected to 2 pulses of 2000 volts between aluminium electrodes spaced approximately 1 cm apart using a conventional electroporation apparatus having a capacitance of 14 μF. Cells were then returned to ice for 5–10 minutes, resuspended in non-selective growth medium (DMEM) and distributed among 24-well culture trays. Selective medium (G-DMEM) was added subsequently as described below.

EXAMPLE 1

Preliminary experiments indicated that the plasmid pSV-LGS1 which has been used successfully as a selectable marker in CHO-K1 cells (see EP-A-0 256 055) could not be introduced into NSO cells to confer glutamine independent growth at efficient rates. Only a very low frequency of approximately 6 transfected colonies per 10^7 cells was obtained. This should be compared with the transfection frequencies in excess of $1/10^4$ transfected cells which are obtained using the xanthine-guanine phosphoribosyl transferase (gpt) selectable marker gene from pEE6gpt and selecting for resistance to mycophenolic acid in medium containing xanthine, hypoxanthine and thymidine. Preliminary experiments using PCMGS, in which the GS cDNA is expressed from the hCMV-MIE promoter yielded a much higher frequency of glutamine-independent colonies and pSV2GS, which utilises the SV40 early region promoter, yielded an intermediate transfection frequency.

In order to establish a suitable protocol for consistent use in selection, 20 μg linearised pCMGS and 20 μg of linearised pEE6gpt were mixed and introduced together into 10^7 NSO cells. A separate aliquot of 10^7 cells was "mock" transfected by electroporation without added DNA.

The cells were plated in 24-well plates as described above in 0.5 ml non-selective medium (DMEM) and 24 hours later, selection was applied to wells of the "mock" and DNA transfected plates as follows:

A added 1 ml G-DMEM and left for 7 days before examining plates.

B added 1 ml G-DMEM on day 1, aspirated on day 2 and replaced with fresh G-DMEM.

C added 1 ml G-DMEM containing 200 μM glutamine on day 1, left for 2 more days then aspirated and replaced with G-DMEM.

D left on day 1 and added 1 ml G-DMEM on day 2. On day 3, medium was aspirated and replaced with fresh G-DMEP.

E left on day 1 and aspirated on day 2 and replaced with 1 ml G-DMEM. The wells were aspirated again on day 4 and replaced with fresh G-DMEM.

F mycophenolic acid selection: added 0.5 ml gpt-selective medium and on day 2 added 0.5 ml of 2× gpt-selective medium.

The number of surviving colonies in each of at least 3 wells for each selective protocol was scored 7–10 days after transfection and the mean results are shown in Table 2.

TABLE 2

Selective	Mean No. colonies/10^6 cells plated	
Protocol	MOCK	pCMGS + pEE6gpt
A	0	130
B	0	3
c	0	72
D	0	8
E	0	8
F	0	115

From these results it appears that protocol A (addition of 1 ml G-DMEM one day after transfection) provides the highest survival of transfected colonies and the frequency obtained when selecting for the introduced GS gene is equal to the transfection efficiency measured by selection for the gpt gene (Protocol F). Addition of a small amount of glutamine and aspiration after 2 more days, to replace with G-DMEM alone, (protocol C) provides the next highest frequency of glutamine independent colonies. However, removing the medium by aspiration and hence complete removal of glutamine (protocols B, D and E) severely reduces the number of surviving colonies. It can thus be seen

that progressive depletion of glutamine in the medium leads to an enhanced selection procedure. Therefore protocol A was used in all subsequent experiments.

A strong promoter such as the hCMV-MIE promoter is likely to provide a high level of GS expression and hence will require a high level of MSX to select for gene amplification.

In order to determine whether a weaker promoter than the hCMV-MIE promoter-enhancer can be used to express a GS cDNA to obtain glutamine-independent transformants, 40 μg lincarised pSV2BamGS, which uscs the SV40 Early region promoter for GS expression, was introduced into NSO cells and selected using Protocol A. The results are shown in Table 3.

TABLE 3

DNA Transfected	Selection Protocol	Mean No. colonies/10^6 Cells
20 μg pCMGS	A	100
20 μg pEf6gpt	F	40
40 μg pSV2BamGS	A	20
"Mock"	A	0

Thus it appears that the GS-transcription unit in SV2BamGS can indeed be used as a selectable marker in NSO cells but confers glutamine independence at a lower frequency than does PCMGS.

In order to test whether the GS gene in pSV2BamGS can be used as a selectable marker for the introduction of heterologous DNA into NSO cells, three different plasmids were constructed which contain different non-selectable linked genes all under the control of the hCMV MIE promoter-enhancer. These are pcLc2GS, which contains the chimeric B72.3 immunoglobulin light-chain gene; pTIMPGS, which contains the gene for tissue inhibitor of metalloproteinasc (TIMP); and pAb2GS which contains both heavy and light chain genes for the chimeric B72.3 monoclonal antibody. Each was introduced into NSO cells as a linear plasmid using 40 μg of pcLc2GS and pTIMPGS and 80 μg of pAb2GS per 10^7 cells transfected. The transfection frequency was $4/10^7$ cells for pAb2GS and no colonies were obtained with either of the other two plasmids. The 4 colonies obtained from transfection with pAb2GS were grown in bulk culture and spent culture supernatant analysed by Western blotting using anti-heavy and anti-light chain antibodies. The result is shown in FIG. 1. It is clear that all 4 clones secrete both heavy and light chains but at very low level (undetectable by enzyme-linked immunosorbent assay). Thus the GS-transcription unit from pSV2BamGS can be used as a selectable marker to introduce heterologous genes into NSO cells but the presence of such genes in these particular plasmid constructions seems to reduce substantially the frequency with which transfected colonics can be isolated. It is likely that this is due to an interference between the various transcription units on the plasmid. Hence only those few colonies in which the genes upstream of the GS-transcription unit are for some reason exceptionally poorly expressed, can yield sufficient GS for survival.

In order to test whether the position of genes on the vector was indeed responsible for the dramatic reduction in frequency of transformation to glutamine-independent growth, plasmids were constructed in which the GS gene transcription is upstream of a cL chain gene instead of downstream as in the previous experimcnts. The two plasmids choscn were pCMGScLc and pSV2GScLc, in which the GS gene is under the control of the hCMV-MIE promoter and SV40 early region promoter, respectively. These plasmids were introduced into NSO cells and transfectants selected using Protocol A. The number of colonies obtained is shown in Table 4.

TABLE 4

Plasmid	No. Colonies/10^6 cells
pCMGS	250
pCMGS.cLc	300
pSV2.GS	18
pSV2GS.cLc	9
pcLc2GS	0

These results show that whereas pcLc2GS, in which the GS gene is downstream of the cl.c gene, yields no glutamine-independent colonies, the equivalent plasmid, pSV2GScLc, which has the gene order reversed, with the GS gene upstream, yields a transformation frequency comparable to that obtained using the GS gene alone (pSV2GS).

This study suggests that any interference of the SV40 early promoter used to express GS is reduced by placing the strong hCMV-MIE promoter downstream. The results in Table 4 also show no significant difference in the transformation efficiency obtained with pCMGScLc compared with pCMGS, again indicating no interference with GS expression.

The transfectants obtained with plasmids pCMGScLc and pSV2GscLc were assayed for cL chain secretion using an ELISA assay for human kappa chain antigen activity in spent culture medium. All culture wells from the pCMGScLc transfection, each containing many transfected colonies, did indeed secrete significant amounts of antigen. Seven out of ten wells from the pSV2GScLc transfection, again containing multiple colonies, also secreted detectable levels of light chain.

This is marked contrast to the results obtained with plasmids in which the GS gene is downstream of the second gene controlled by the hCMV-MIE promoter and demonstrates that the GS gene can be used as an effective selectable marker in this cell type, provided that the plasmid is appropriately designed.

pSV2GS is a particularly suitable vector into which heterologous genes may be inserted and pSV2GScLc is shown in FIG. 2.

In order to test which of the additives present in G-DMEM is essential for the growth of GS transfectants, pooled transfected cells containing the plasmid pCMGScLc were distributed among wells of a 24 well tissue culture tray in DMEM with 10% dialysed foetal calf serum and containing all possible combinations of the following additives at the concentrations present in G-DMEM: a) non-essential amino acids; b) glutamate; c) asparagine; d) adenosine, guanosine, cytidine, uridine and thymidine. Growth was scored after four days and the results are shown in Table 5.

TABLE 5

Medium Additions	Growth
None	-
abcd	++
abd	++
ab	++
ac	++
ad	++
a	++

TABLE 5-continued

Medium Additions	Growth
b	–
bc	++
bd	–
bcd	++
c	++
cd	++
d	—
bd4	–

From this it is clear that 500 μM asparagine is sufficient to sustain growth of GS transfectants in the absence of any other of the additives in G-DMEM. Surprisingly, glutamate, the substrate for GS, will not sustain growth of these cells, even when the concentration is raised to 2 mM. Non-essential amino acids can be used instead of 500 μM asparagine to support growth of these transfectants, but as this additive contains 100 μM asparagine, it is possible that this concentration of asparagine alone is sufficient to support growth.

It is clear that the NSO cell line must contain insufficient active GS-enzyme to permit growth in the glutamine-free medium used here and that a plasmid such as PCMGS yields sufficient GS when expressed in these cells to allow glutamine independent growth. Clones expressing a GS gene under the control of a weaker promoter, such as the SV40 Early promoter, on average express less GS enzyme and only a proportion of transfectants can survive in the glutamine-free medium.

In order to test whether GS-vectors can be used to confer glutamine-independent growth on other lymphoid cell lines, the growth of three additional cell lines in glutamine-free media was investigated. P3-X63Ag8.653 (a mouse myeloma) was also found to be completely incapable of growth in G-DMEM. When 10^7 cells were plated-out in a 24-well plate and selected using Protocol A, no glutamine independent variants were isolated. In contrast the non-secreting mouse hybridoma SP2/0 generated variants able to grow in G-DMEM at a frequency of approximately $1/10^5$ cells plated. Since the transfection frequency in this cell line (eg using pEE6gpt) is also about $1/10^5$ cells plated, this cell line is unsuitable as a host for GS selection using this protocol.

The rat non-secreting hybridoma YB2/0 also yielded glutamine-independent variants at an even higher frequency, estimated at $1/10^5$, making this cell line unsuitable for use with the GS-selection protocol developed above. A glutamine-independent variant of YB2/0 was cloned and a clonal cell line, designated YOG- F10, was grown in bulk culture and stored as frozen stocks in liquid nitrogen. A similar cloned cell line, a glutamine-independent variant of SP2/0, termed SPG2-E4 was also stored in liquid nitrogen. Such cell lines will be suitable for the introduction of vectors containing GS genes by methods described in EP-A-0 256 055.

In order to test whether selection for glutamine-independent transformants can be used to introduce plasmid vectors into P3-X63Ag8.653 cells, 40 μg linear pCMGSgpt (a vector containing both the GS-transcription unit from PCMGS and the gpt gene from pEE6gpt) was introduced by electroporation into 10^7 P3-X63Ag8.653 cells. The results are shown in Table 6.

TABLE 6

Transfection of P3-X63-Ag8.653		
PLASMID	SELECTION PROTOCOL	NO. COLONIES/10^5 CELLS
pCMGS.gpt	A	120
	F	24
"Mock"	A	0
	F	0

Thus the hCMV-GS transcription unit and selection Protocol A chosen for NSO cells can be used to obtain glutamine-independent P3-X63Ag8.653 at a frequency which is at least as great (possibly higher) than obtained using gpt selection. It should be noted that interference ("promoter occlusion") between the two transcription units could account for the lower frequency at which gpt-selected colonies arise.

The myeloma cell lines NSO and P3-X63Ag8.653 have been successfully transformed to glutamine independent growth by transfection with GS-expression plasmids. In contrast, two hybridoma lines, YB2/0 and SP2/0 generate glutamine-independent variants at too high a frequency for GS-plasmids to be used in this way as selectable markers in these cells. It is also demonstrated here that a GS-expression plasmid such as pSV2GS can be used to introduce non-selected genes such as the genes encoding the B72.3 chimeric antibody into NSO cells. The arrangement of genes on the plasmid have marked effects on the expression levels attained from these genes and it will be important to take this into account in the design of optimal expression vectors. Transcription from a strong promoter such as the hCMV-MIE promoter-enhancer should not be permitted to proceed towards a gene expressed from a weaker promoter, such as the SV40 Early promoter unless the two genes are separated, e.g. by a transcription termination signal.

EXAMPLE 2

In order to confirm that the GS-transcription units in plasmids such as pCMGS and pSV2BamGS can act as amplifiable selectable markers in a myeloma cell line, the copy number of vector DNA introduced into NSO cells by electroporation was analysed before and after selection for GS-gene amplification using methionine sulphoximine (MSX).

NSO cells were transfected with pSV2BamGS or PCMGS as described above and pools of transfected colonies (at least 20 colonies from each transfection) were expanded in culture to form cell lines SV2GSNSO and CMGSNSO. These two cell pools were then distributed among the wells of a 24-well cell culture tray at a cell density of approximately cells/well in G-DMEM medium. MSX was added to the wells to final concentrations ranging between 20 and 80 μM. After incubation for several days, extensive cell death was observed in all wells and, after 2-3 weeks, MSX-resistant colonies were visible at concentrations of MSX up to 60 μM for the SV2GSNSO cell line and up to 80 μM for the CMGSNSO cell line. Cells isolated at these concentrations of MSX were replated at approximately 10^5 cell/well in 24-well trays and selection reapplied at concentrations up to 100 μM. In both cases there was considerable cell survival at 100 μM. 100 μM MSX-resistant cell pools were expanded in culture and total genomic DNA prepared from initial transfectants and from cell pools resistant to 100 μM MSX.

DNA samples were digested with BglI and BglII restriction enzymes and a Southern blot of the DNA samples was probed with the 0.5 kb 5' PstI GS-cDNA fragment from pGSC45 [7]. The Southern blot analysis is shown in FIG. 3. The DNA probe cross-hybridises with the endogenous mouse GS-gene in NSO cells and this is seen as a fragment of approximately 2.8 kb and one of approximately 6 kb in all transfected cell lines, as well as in non-transfected NSO control DNA. These bands serve as an internal control for loading of the same amount of DNA in each track on the gel. In cells transfected with pSV2BamGS, a 1.2 kb BglII fragment is also detected, which is of the size predicted for vector DNA (from a BglI site in the SV40 promoter to the BglII site at the 3' end of the GS cDNA). By comparison with known amounts of vector DNA loaded in adjacent tracks, the average vector copy number in the SV2GSNSO pool is estimated to be approximately 1 copy/cell. After selection in 100 μM MSX, the copy number is increased to an average of about 5 copies/cell.

Similarly, introduction of pGiMGS into NSO cells has led to the appearance of the predicted 2.1 kb vector fragment in the Southern blot of CMGSNSO DNA at an average level of approximately 1 copy/cell. Selection with 100 μM MSX has led to an increase in copy-number to approximately 10 copies/cell.

This experiment clearly indicates that pCMGS and pSV2BamGS introduced into NSO cells can be amplified by selection with MSX. No amplification of the endogenous mouse GS genes could be detected.

EXAMPLE 3

In order to test the efficacy of the MSX selection procedure described in Example 1 for the overproduction of recombinant product, the expression of B72.3 cL-chain introduced into NSO cells using GS selection was measured before and after selection with MSX. The transfection frequency was approximately 2×10^{-4} colonies/cell transfected for pSV2GSCLC and approximately 10^{-5} for pCMG-ScLc.

Two NSO-derived cell lines transfected with pSV2GScLc and two cell lines transfected into pCMGScLc, all secreting high levels of cL chain were first recloned by limiting dilution. The expression of cL chain from the 4 highest producing independent clones isolated were estimated by ELISA in comparison with a purified standard preparation of B72.3 cL-chain which had been quantitated by optical density (OD_{280}) measurement. The results are shown in Table 7, together with the production rates of MSX-resistant pools derived from these cloned cell lines as described in Example 1. Pools of resistant colonics were isolated at 40 μM NSX for three of the cell lines and at 100 μM MSX for cell line C2-27.

TABLE 7

Rates of secretion of cL-chain of B72.3 from transfected NSO cell lines before and after selection for GS-gene amplification.

	SECRETION RATES (pg/cell/day)		
CELL LINE	TRANS-FECTANT	AMPLIFIED POOL	AMPLIFIED CLONE
SVGSCLc-B4.24	4.6	13	20
-C2.27	0.4	3	
CMGS.cLc-9/6	1.5	0.2	

TABLE 7-continued

Rates of secretion of cL-chain of B72.3 from transfected NSO cell lines before and after selection for GS-gene amplification.

	SECRETION RATES (pg/cell/day)		
CELL LINE	TRANS-FECTANT	AMPLIFIED POOL	AMPLIFIED CLONE
-13/7	3	9	

Thus significant increases in productivity are seen for 3 out of the 4 cell lines as a result of selection for resistance to MSX.

The cell-pool with the highest average secretion rate, B4.24 (40 μM) was cloned by limiting dilution and a clonal cell line was isolated which secreted cL chain at a rate of 20 pg/cell/day. This represents an increase of greater than 4-fold relative to the expression level of the original transfected clone, indicating that selection for GS gene amplification does indeed lead to improved production of the desired recombinant product.

EXAMPLE 4

NSO cells were transfected with a GS-vector containing genes for both the cH and cL chains of the B72.3 antibody in order to express a complete immunoglobulin molecule from a myeloma cell line.

Plasmid pST-6 contains the cH and cL chain cDNA coding sequences, each under the control of an hCMV promoter and a SV40 poly A signal and the SV40 early-GS transcription unit from pSV2BamGS. This plasmid was linearised with SalI and introduced into NSO cells by electroporation as described above. The transfection frequency was approximately 2×10^{-5} colonies/cell transfected. Transfectant lines were screened for secretion of functional antibody in a binding assay using antigen-coated plastic plates.

The amount of antibody secreted was then quantitated by an ELISA designed to detect only assembled immunoglobulin. The rates of secretion of assembled antibody for the three of the highest expressing cloned cell lines and MSX-resistant pools derived from them are shown in Table 8.

TABLE 8

	SECRETION RATES (pg/cell/day)		MSX* Conc.
CELL LINE	TRANSFECTANT	AMPLIFIED POOL	(μM)
6A1	1.5	3.2	100
6-11D3	1.2	0.6	80
6-11D5	0.4		80

*MSX concentration is the concentration to which the amplified pools are resistant.

Thus in one of the three cell lines analysed, the expression of antibody is increased significantly by selection for GS-gene amplification. The results in Table 8 obtained using the plasmid pST-6 are markedly different from those obtained using pAb2GS described in Example 1, in which the frequency with which transfectant colonies can be isolated is markedly reduced due to promoter interference. In transfection with pAb2GS, only those few colonics in which the immunoglobulin genes upstream of the GS-gene are exceptionally poorly expressed yield sufficient GS for sur-

vival. Consequently the transfectants isolated were poor antibody producers. Using pST-6, it is possible to isolate cell lines secreting much higher levels of antibody. It will be apparent to those skilled in the art that additional alterations to the arrangement of genes on the vector will further reduce transcriptional interference with further beneficial effects of antibody-expression.

EXAMPLE 5

pEE690KGS was the introduced into the myeloma cell line NSO by electroporation as described in Example 1. Transfectants were selected by growth on glutamine free medium (G-DMEM) after plating out in a 96 well plate. The transfection efficiency was approximately 1 in 10^5 cells transfected.

Wells of the cell culture trays containing single colonies were analysed for secretion of the 90 kD fibrinolytic enzyme by fibrin agar plate assay [8]. Of 33 wells assayed, 26 were clearly positive for fibrinolytic activity. Positive transfectants were then expanded in culture for analysis of the rate of product secretion.

Cell lines isolated in this way secreted between 0.02 and 3.75 pg/cell/day as estimated by fibrin plate assay in comparison with a tissue plasminogen activator (tPA) standard. The 6 cell lines with the highest secretion rates are shown in Table 9. The fibrinolytic activity was shown to have the expected molecular weight of 90 kD by zymography, carried out according to Dodd [9].

The five cell lines with the highest secretion rates were then selected for GS gene amplification using MSX applied in the range 20–80 μM, as described in Example 2. The specific production rates of MSX resistant cell-pools were determined using the fibrin plate assay and the results are shown in Table 10.

TABLE 9

Production rates of initial transfectant cell lines secreting a 90kD fibrinolytic enzyme.

Line	pg/cell/day
1	3.5
5	0.75
9	1.4
12	1.5
22	1.7

TABLE 10

Production rates of cell lines secreting 90 kD fibrinolytic enzyme after one round of selection for vector amplification

		Secretion rates	
Line	MSX Conc	Pool pg/cell/day	Clone pg/cell/day
1	40 μM	5.8	4.5
5	60 μM	1.2	
9	80 μM	4	10.5
12	80 μM	5.65	
22	40 μM	4.75	6
23	60 μM	7.5	

A second round of selection for vector amplification was then carried out by further increasing the MSX concentration using the first round amplified pools as described in Example 2. Again specific production rates were determined and are shown in Table 11. This second round of selection led to further increases in productivity in each of the pooled cell lines tested, although when clones from these lines were analysed, the highest producers secreted approximately 10 pg/cell/day whether the clones were isolated after the first or the second round of selection for GS amplification. This may indicate that this level of product secretion is saturating for these cell lines.

These results indicate that the level of expression is increased by selection of MSX resistant variants. A clone of line 9 selected for resistance to 80 μM MSX has a secretion rate increased from 1.4 pg/cell/day to 10.5 pg/cell/day after selection for GS gene amplification, an increase of 7.5 fold.

TABLE 11

Specific Production Rate for 2nd Round Amplified Lines

Line	[MSX]	Pool pg/cell/day	Clone pg/cell/day
$_{1}$40	100 μM	7.75	
$_{5}$80	300 μM	6.5	
$_{1}$280	400 μM	7.2	10
$_{22}$40	300 μM	6.4	8
23^{80}	200 μM	8.0	

It will be appreciated that the present invention has been described above by way of illustration only and that modifications in detail can be made using the skilled person's ordinary knowledge of the art without departing from the scope of the present invention.

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What is claimed is:

1. A method of conferring glutamine independence to a myeloma cell line, comprising transforming said myeloma cell line with a vector comprising a GS gene and a gene or genes encoding a protein(s) heterologous to said myeloma cell line, wherein the genes are arranged such that said GS gene can be expressed and glutamine independent myeloma colonies can be produced.

2. The method of claim **1**, wherein said gene or genes encoding the protein(s) heterologous to said myeloma cell

line comprises a relatively strong promoter, and wherein said GS gene comprises a relatively weak promoter located upstream of said gene or genes encoding the protein(s) heterologous to said myeloma cell line so that transcription of the heterologous gene or genes does not run through the GS gene.

3. The method of claim 2, wherein said relatively weak promoter is the SV40 early region promoter and said relatively strong promoter is the hCMV-MIE promoter.

4. The method of claim 1, wherein said gene or genes encoding the protein(s) heterologous to said myeloma cell line comprises a relatively strong promoter, and wherein said GS gene comprises a relatively weak promoter that directs expression in the opposite direction to that of said gene or genes encoding the protein(s) heterologous to said myeloma cell line.

5. The method of claim 4, wherein said relatively weak promoter is the SV40 early region promotor and said relatively strong promoter is the hcMV-MIE promoter.

6. The method of claim 4, wherein said vector comprises a GS gene that comprises a weak promoter, and wherein said gene or genes encoding the protein(s) heterologous to said myeloma cell line comprises an Ig heavy chain gene having a strong promoter and an Ig light chain gene having a strong promoter, wherein said strong promoter of said light chain gene is oriented in the opposite direction to said promoters of said GS and heavy chain genes, and wherein said Ig heavy chain gene is downstream from said GS gene.

7. The method of claim 1, wherein said vector comprises a GS gene that comprises a weak promoter, and wherein said gene or genes encoding the protein(s) heterologous to said myeloma cell line comprises an Ig heavy chain gene having a strong promoter and an Ig light chain gene having a strong promoter, wherein said strong promoter of said Ig light chain gene is orientated in the opposite direction to said promoters of said GS and heavy chain genes, and wherein said Ig heavy chain gene is downstream from said GS gene so that transcription of the heterologous gene does not run through the GS gene.

8. The method of claim 1, wherein said GS genc comprises a weak promoter, wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises an Ig light chain gene having a strong promoter and an Ig heavy chain gene having a strong promoter, wherein said GS gene, Ig light chain gene, and Ig heavy chain gene are transcribed in the same direction, and wherein said GS gene is located upstream of said Ig light chain gene and said Ig heavy chain gene so that transcription of the heterologous gene(s) does not run through the GS genc.

9. The method of claim 1 wherein the GS gene is expressed from an SV40 early region promoter.

10. The method of claim 1 wherein the GS gene is expressed from an hCMV-MIE promoter.

11. The method of claim 9 wherein the GS gene and promoter are derived from pSV2GS.

12. The method of claim 10 wherein the GS gene and promoter are derived from pCMGS.

13. The method of claim 1 wherein all genes are expressed from the same type of promoter.

14. The method of claim 13 wherein the type of promoter is the hCMV-MIE promoter.

15. The vector of claim 14 wherein the vector is derived from pCMGS.

16. The vector of claim 8 wherein the vector is pSV2GSeLeeHe or pST6.

17. A method of selecting myeloma cells transfected with a vector comprising a GS genc and a genc or gencs encoding a protein(s) heterologous to said myeloma cells, comprising:

(i) plating transfected cells in one volume of non-selective medium containing glutamine;

(ii) after 24 hours, adding two volumes of glutamine-free medium; and

(iii) recovering myeloma colonies after 7 days incubation.

18. A method of conferring glutamine independence to a lymphoid cell line, comprising transforming said lymphoid cell line with a vector comprising a GS gene and a gene or genes encoding a protein(s) heterologous to said lymphoid cell line, wherein the genes are arranged such that said GS gene can be expressed and glutamine independent lymphoid colonies can be produced.

19. The method of claim 18, wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises a relatively strong promoter, and wherein said GS gene comprises a relatively weak promoter located upstream of said gene or genes encoding the protein(s) heterologous to said lymphoid cell line so that transcription of the heterologous gene or genes does not run through the GS gene.

20. The method of claim 19 wherein said relatively weak promoter is the SV40 early region promoter and said relatively strong promoter is the hCMV-MIE promoter.

21. The method of claim 18, wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises a relatively strong promoter, and wherein said GS gene comprises a relatively weak promoter that directs expression in the opposite direction to that of said gene or genes encoding the protein(s) heterologous to said lymphoid cell line.

22. The method of claim 21, wherein said relatively weak promoter is the SV40 early region promoter and said relatively strong promoter is the hCMV-MIE promoter.

23. The method of claim 21, wherein said vector comprises a GS gene that comprises a weak promoter, and wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises an Ig heavy chain gene having a strong promoter and an Ig light chain gene having a strong promoter, wherein said strong promoter of said light chain gene is oriented in the opposite direction to said promoters of said GS and heavy chain genes, and wherein said Ig heavy chain gene is downstream from said GS gene.

24. The method of claim 18, wherein said vector comprises a GS gene that comprises a weak promoter, and wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises an Ig heavy chain gene having a strong promoter and an Ig light chain gene having a strong promoter, wherein said strong promoter of said Ig light chain gene is orientated in the opposite direction to said promoters of said GS and heavy chain genes, and wherein said Ig heavy chain gene is downstream from said GS gene so that transcription of the heterologous gene does not run through the GS gene.

25. The method of claim 18, wherein said GS gene comprises a weak promoter, wherein said gene or genes encoding the protein(s) heterologous to said lymphoid cell line comprises an Ig light chain gene having a strong promoter and an Ig heavy chain gene having a strong promoter, wherein said GS gene, Ig light chain gene, and Ig heavy chain gene are transcribed in the same direction, and wherein said GS genc is located upstream of said Ig light chain gene and said Ig heavy chain gene so that transcription of the heterologous gene(s) does not run through the GS gene.

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